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# Cadaverine Protects *Vibrio vulnificus* from Superoxide Stress

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**Abstract** An electron paramagnetic resonance (EPR) signal characteristic of the 5,5'-dimethyl-1-pyrroline-*N*-oxide (DMPO)-OH spin adduct, which is formed from the reaction of DMPO with superoxide radicals generated by xanthine oxidase-mediated reaction, was significantly reduced by the cadaverine or *Escherichia coli* Mn-containing superoxide dismutase (MnSOD). Likewise, cytochrome *c* reduction by superoxide was inhibited by cadaverine, and the inhibition level increased in proportion to the level of cadaverine. The *cadA* mutant of *Vibrio vulnificus*, which does not produce cadaverine because of the lack of lysine decarboxylase, exhibits less tolerance to superoxide stress in comparison with wild type. The results indicate that cadaverine scavenges superoxide radicals, and protects cells from oxidative stress.

**Key words:** *Vibrio vulnificus*, lysine decarboxylase, cadaverine, *cadBA*

*Vibrio vulnificus* is a pathogen causing foodborne gastroenteritis (for a review, see Reference 1). The bacterium is sensitive to low pH but may travel through the acid environment of the stomach to colonize the intestinal lumen. The ability to tolerate gastric acidity is necessary for the pathogen to cause foodborne infections. The physiological responses to cope with the acidic stress also contribute to the virulence of many enteric bacteria including *Escherichia coli* and *Vibrio cholerae* [12, 16, 26].

The generation of amines (cadaverine, agmatine, and putrescine) and $CO_2$ by amino acid decarboxylases in the presence of their respective substrates (lysine, arginine, and ornithine, respectively) is one of the multiple effects under acid stress (for a review, see Reference 27). When *Vibrio vulnificus* is exposed to low pH, lysine decarboxylase (CadA) is induced to catalyze cadaverine formation from lysine that is transported into the cell through the lysine-cadaverine antiporter (CadB) [20, 21]. The neutralization of external pH by cadaverine has been known to protect *V. vulnificus* from acid stress [21]. The induction of *cadBA* transcription has been well illustrated as an acid pH-dependent response in *E. coli* and *V. cholerae* as well [17, 19]. CadC, a membrane-bound protein whose gene lies upstream from *cadBA*, has been identified as a positive regulator of *cadBA* expression [17, 19, 22].

Polyamines are associated with many biochemical processes, such as the regulation of gene expression, the stabilization of chromatin, and the prevention of DNA damage [6, 27, 29]. Polyamines, of which spermine may directly scavenge oxygen radicals [7], have been also known to protect cells from the toxic effects of reactive oxygen such as hydrogen peroxide, singlet oxygen, and oxygen radicals [2, 6, 18, 28]. Since we have shown that superoxide stress also builds up in *V. vulnificus* at low pH [10], we next determined whether cadaverine is also involved in relieving oxidative stress. Indeed, cadaverine is found to directly scavenge superoxide radicals, as verified from the *in vitro* EPR and cytochrome *c* reduction assays. Furthermore, *V. vulnificus* mutant unable to form cadaverine also shows lower tolerance to superoxide stress.

*V. vulnificus* was grown at 30°C in Luria-Bertani (LB) medium [24] supplemented with 2% (wt/vol) NaCl (LBS, pH 7.5) [8]. *E. coli* was grown at 37°C in LB. When appropriate, antibiotics were added at concentrations as described previously [10]. Cell growth was measured as described previously [10, 25].

EPR was measured on a Bruker model EMX spectrometer. A standard superoxide-generating mixture of xanthine (100 μM) and xanthine oxidase (0.004 U/ml) was prepared in 100 mM potassium phosphate (pH 7.4) buffer [4] containing 150 mM DMPO (Sigma, St. Louis, MO, U.S.A.). Desferrioxamine (100 μM) was included to chelate iron impurities [5] The mixture was incubated in the dark for 10 min at 30°C and placed in a quartz EPR flat cell. The

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spectra were recorded at ambient temperature. The EPR-settings were as follows: modulation amplitude 0.32 G, time constant 0.16 s, scan time 60 s, modulation frequency 100 kHz, microwave power 20 mW, and microwave frequency 9.76 GHz [23].

Cytochrome *c* reduction assay was performed in 50 mM potassium phosphate (pH 7.8) buffer containing 0.1 mM EDTA and 10 μM cytochrome *c*. Superoxide was generated from the mixture of xanthine (100 μM) and xanthin oxidase (0.004 U) as described above. Cytochrome *c* reduction was recorded by an increase of absorbance at 550 nm using an extinction coefficient of 21,000 $M^{-1}cm^{-1}$ as described previously [13, 15].

Tolerance to oxidative stress was examined essentially as described previously [10]. Cells were grown to late logarithmic phase ($A_{600}$, ~4.0) in LBS (pH 7.5), harvested, washed with LBS (pH 5.0), and suspended in the same LBS (pH 5.0) to a final concentration of $10^5$ colony forming units (CFU)/ml. Cells were grown for 4 h for the induction of *cadBA* transcription. Thereafter, cells were harvested, washed with phosphate-buffered saline (PBS, pH 7.5) supplemented with 3 mM methyl viologen (MV), and suspended in the same buffer to a final concentration of $10^5$ CFU/ml. A control experiment was performed, in which PBS (pH 7.5) without MV was used. Cell suspensions were incubated for 1.5 h at 30°C with shaking as described for cell growth [10]. Samples were then taken and viable counts (CFU/ml) were determined by plating dilutions of cells on LBS (pH 7.5) agar plates. Survival was expressed as percentage of the initial CFU. Data shown are representatives of triplicate experiments, yielding similar results; the averages of three independent experiments are shown.

**Superoxide Reaction with Spin Trap DMPO is Inhibited by Cadaverine.** It was determined whether superoxide is directly scavenged by cadaverine. A spin trap DMPO is included in the xanthine oxidase reaction, the most widely used biochemical source of superoxide [4]; DMPO reacts with superoxide to reveal a signal characteristic of the DMPO-OH spin adduct with a quartet signal showing intensity ratios of 1:2:2:1 and hyperfine coupling constants of $\alpha^N = \alpha\beta^H = 14.89$ G in EPR spectroscopy (Fig. 1). DMPO-OH is formed through spontaneous decay of the superoxide spin adduct DMPO-OOH [3]. The signal was totally abolished by the addition of *E. coli* MnSOD (100 U/ml). Likewise, it was significantly reduced by cadaverine (1 mM) in the reaction mixture (Fig. 1). The formation of uric acid, which is an oxidized product of xanthine by xanthine oxidase, was examined by measuring $A_{293}$ of the reaction mixture [14], and was not changed irrespective of the presence of cadaverine up to 5 mM (data not shown). Thus, the xanthine oxidase reaction generating superoxide was not affected by cadaverine. The results argue that cadaverine is capable of scavenging superoxide radicals.

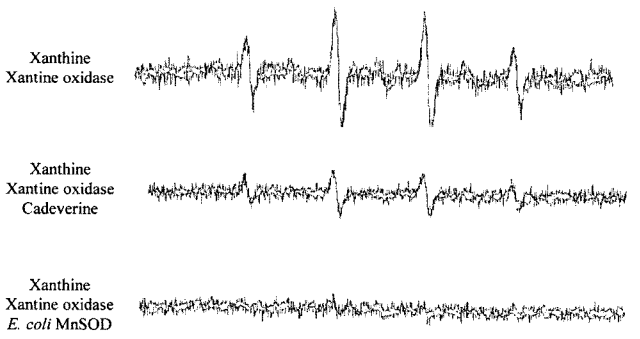


**Fig. 1.** Superoxide reaction with DMPO in the presence of cadaverine.
A signal characteristic of the DMPO-OH spin adduct, which is formed by the reaction of DMPO with superoxide radical, was examined after DMPO was included in the xanthine oxidase reaction as described in the text. Top, no addition; middle, cadaverine 1 mM; bottom, *E. coli* MnSOD 100 U/ml of reaction mixture.

**Cytochrome *c* Reduction by Superoxide is Inhibited by Cadaverine in a Dose-Dependent Way.** The involvement of cadaverine in relieving oxidative stress was also determined using the cytochrome *c* reduction method (Fig. 2). The xanthine oxidase reaction generates superoxide radicals, which in turn reduce cytochrome *c*. If cadaverine scavenges superoxide free radicals, the cytochrome *c* reduction by the radicals would be inhibited. The rate of cytochrome *c* reduction in this work was taken as the rate of formation of superoxide radical, since its reduction was totally abolished by *E. coli* MnSOD (100 U/ml). The cytochrome *c* reduction was measured in the presence of increasing concentrations of cadaverine. The reduction rate of

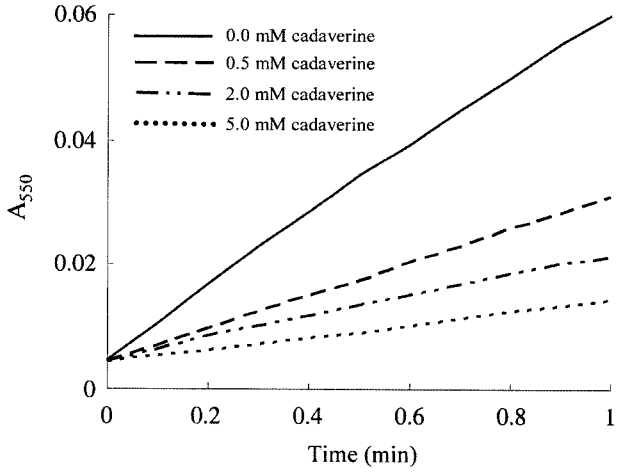


**Fig. 2.** Cytochrome *c* reduction assay in the presence of increasing concentrations of cadaverine.
Xanthine oxidase reaction was performed in the presence of 20 μM cytochrome *c*, which is also supplemented with cadaverine up to 5 mM. Cytochrome *c* reduction was recorded by an increase of $A_{550}$. The rate of cytochrome *c* reduction in this work was taken as the rate of formation of superoxide radical, since its reduction was totally abolished by *E. coli* MnSOD (100 U/ml) (data not shown).

cytochrome *c* was lowered as the level of cadaverine was increased (Fig. 2). The concentration of cadaverine inhibiting the cytochrome *c* reduction by 50% was determined to be approximately 0.5 mM, which appears to be consistent with the result shown with EPR analysis. Thus, the *in vitro* results shown in this work demonstrate that cadaverine effectively removes superoxide stress, possibly by scavenging the radicals.

***cadA* Mutant Shows Less Tolerance to Superoxide Stress.** Cadaverine formation in *V. vulnificus* is catalyzed by lysine decarboxylase, which is induced in acidic environment [21]. Cadaverine is excreted through a lysine-cadaverine antiporter (CadB) and neutralizes the external medium to protect cells from the acid stress. Although cadaverine is shown to scavenge superoxide *in vitro*, it remains to be determined whether it is also effective in protecting cells from oxidative stress. LBS (pH 5.0) was inoculated with the exponentially growing cells. The lysine decarboxylase expression of the wild-type cell at low pH was induced to show the enzyme activity between 20–55 units $A_{600}^{-1}$, whereas no enzyme induction was observed in the *cadA* mutant. The wild type and *cadA* mutant of *V. vulnificus* were then transferred to PBS (pH 7.5) supplemented with 3 mM MV. A transfer into PBS (pH 7.5) without MV was included as a control. Viable cell counts were performed as described above, and survival was expressed as percentage of the initial CFU.

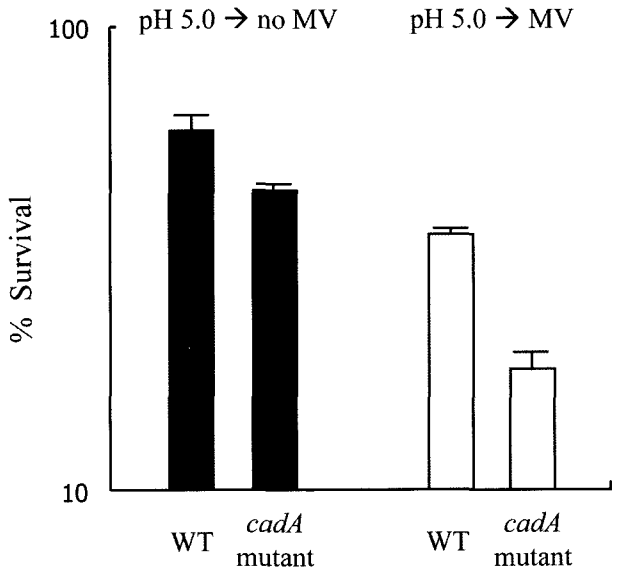


**Fig. 3.** Tolerance of *cadA* mutant to superoxide stress.
Wild type (AR) [11] and *cadA* mutant [21] grown in LBS (pH 7.5) were inoculated into LBS (pH 5.0), and incubated for 4 h for the induction of *cadBA* transcription. Thereafter, both wild type and *cadA* mutant were transferred to PBS (pH 7.5) supplemented with 3 mM MV (pH 5.0 → MV). Transfer of the cells into PBS (pH 7.5) without MV was included as a control (pH 5.0 → no MV). Viable cell counts were performed 1.5 h after shaking incubation in the buffer. The percent survival is shown in logarithmic scale and denotes the percentage of the initial CFU ($10^5$ $ml^{-1}$). Error bars correspond to the standard deviations of the means.

An approximately 40% decrease in survival of *cadA* mutant was observed in comparison with wild type under non-oxidative stress conditions (Fig. 3, pH 5.0 → no MV). However, the mutant showed the survival decrease by approximately 60% compared with wild type under oxidative stress conditions (Fig. 3, pH 5.0 → MV). The diminished tolerance of the *cadA* mutant was complemented with pRKCadA (data not shown, but the same as wild-type shown under the oxidative stress conditions of Fig. 3), in which 2.5-kb HindIII-XhoI DNA including *cadA* was cloned in the same orientation as the *lac* promoter of pRK415 [9]. Thus, the results indicate that the lysine decarboxylase expression of *V. vulnificus* is responsible for the protection of the cells from oxidative stress, possibly by scavenging superoxide radicals with cadaverine.

Taken together, cadaverine is shown to scavenge superoxide radicals from the *in vitro* studies of EPR and cytochrome *c* reduction assays. Furthermore, *V. vulnificus* mutant unable to form cadaverine also shows low tolerance to superoxide stress compared with wild type. Thus, the results indicate that cadaverine protects *V. vulnificus* from the superoxide stress.

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